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IMMUNE STATUS OF FREE-RANGING GREEN TURTLES WITH FIBROPAPILLOMATOSIS FROM HAWAII

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ABSTRACT: Cell-mediated and humoral immune status of free-ranging green turtles (*Chelonia mydas*) in Hawaii (USA) with and without fibropapillomatosis (FP) were assessed. Tumored and non-tumored turtles from Kaneohe Bay (KB) on the island of Oahu and from FP-free areas on the west (Kona/Kohala) coast of the island of Hawaii were sampled from April 1998 through February 1999. Turtles on Oahu were grouped (0–3) for severity of tumors with 0 for absence of tumors, 1 for light, 2 for moderate, and 3 for most severe. Turtles were weighed, straight carapace length measured and the regression slope of weight to straight carapace length compared between groups (KB0, KB1, KB2, KB3, Kona). Blood was assayed for differential white blood cell count, hematocrit, in vitro peripheral blood mononuclear cell (PBMC) proliferation in the presence of concanavalin A (ConA) and phytohaemagglutinin (PHA), and protein electrophoresis. On Oahu, heterophil/lymphocyte ratio increased while eosinophil/monocyte ratio decreased with increasing tumors score. Peripheral blood mononuclear cell proliferation indices for ConA and PHA were significantly lower for turtles with tumor scores 2 and 3. Tumor score 3 turtles (KB3) had significantly lower hematocrit, total protein, alpha 1, alpha 2, and gamma globulins than the other four groups. No significant differences in immune status were seen between non-tumored (or KB1) turtles from Oahu and Hawaii. There was no significant difference between groups in regression slopes of body condition to carapace length. We conclude that turtles with severe FP are imunosuppressed. Furthermore, the lack of significant difference in immune status between non-tumored (and KB1) turtles from Oahu and Kona/Kohala indicates that immunosuppression may not be a prerequisite for development of FP.

Key words: Cell proliferation assay, *Chelonia mydas*, fibropapillomatosis, green turtle, hematology, immunology, protein electrophoresis.

INTRODUCTION

Fibropapillomatosis (FP) is an often-fatal neoplastic disease of marine turtles that causes external fibroepithelial and internal fibromatous tumors. Fibropapillomatosis was first documented in green turtles (*Chelonia mydas*) from Florida (USA) (Smith and Coates, 1938) and has since been reported in loggerheads (*Caretta caretta*) and olive ridleys (*Lepidochelys olivacea*) (Herbst, 1994). While many factors have been suspected to cause FP, recent findings appear to incriminate herpes viruses (Herbst et al., 1998; Quackenbush et al., 1998; Lackovich et al., 1999).

Fibropapillomatosis has a worldwide distribution in marine turtles (Herbst, 1994). In Hawaii (USA), FP is found in green turtles that aggregate in coastal residential foraging pastures of algae and sea grass associated with all islands. The one exception is the Kona/Kohala (west) coast of the island of Hawaii where FP has historically been rarely seen (Balazs, 1991; Balazs et al., 2000b). In Hawaii, FP affects immature green turtles most severely, and the prevalence can reach >50% in some aggregations (Murakawa et al., 2000). Current genetic evidence indicates that green turtles throughout Hawaii belong to a single population (Bowen et al., 1992).

Various investigators have theorized immunosuppression as a contributing or predisposing cause of FP. Aguirre et al. (1995) concluded that green turtles with FP were immunosuppressed based on lymphopenia

and elevated plasma cortisol levels in tumored turtles. Work and Balazs (1999) had similar suspicions based on lymphopenia in green turtles with FP from Molokai. However, investigations to confirm this in free-ranging green turtles by assessing the status of the humoral and cell mediated immune systems are lacking.

Varela (1997) used in vitro cell proliferation assays and plasma electrophoresis to conclude that captive green turtles with FP in Florida were immunosuppressed compared to captive non-tumored animals. Confirming whether immunosuppression is associated with FP in free-ranging green turtles would be of interest for two reasons. First, studying free-ranging turtles might answer if immunosuppression is a predisposing cause of FP. Second, assessing immune status in free-ranging turtles might obviate potential confounding factors, such as different husbandry procedures, often associated with captive animal studies. In this study, we used immunological assays to assess whether significant changes in immune status were associated with FP in free-ranging green turtles in Hawaii.

MATERIALS AND METHODS

We selected two aggregations of immature green turtles. The Kaneohe Bay (21°30′N; 158°95′W) aggregation on the island of Oahu was chosen because of historically high prevalence of FP (Balazs et al., 2000a). Turtles from the Kiholo and Puako Bay aggregations on the Kona/Kohala coast (19°30′N; 156°00′W) of the island of Hawaii were chosen because these areas have been historically free of FP since yearly monitoring started in 1987 (Balazs et al., 2000b). Turtles were captured by hand using SCUBA, snorkel, or diving from slow-moving small boats. Sampling occurred from April 1998 through February 1999.

Straight carapace length (cm) was measured with calipers for each turtle. Gender was not determined because of the absence of any secondary external sex character for immature green turtles. A subset of turtles was weighed (kg) with a spring scale. We assigned a subjective tumor score that was indicative of severity of FP in turtles captured in Kaneohe Bay (Balazs, 1991; Work and Balazs, 1999). Briefly, size of tumors on animals was estimated (cm) and placed into four categories (<1, 1–4, >4–10, and > 10 cm diameter). Based on the number and size of tumors, animals were then assigned a tumor score ranging from 0 (non tumored) to 3 (heavily tumored).

Animals were bled (10 cc) from the cervical sinus (Owen and Ruiz, 1980) with a sterile syringe and 0.9 × 38 mm needle and blood was dispensed into heparinized tubes (18 USP heparin/ml). Blood tubes were stored in an ice chest in the shade until processing in the laboratory 8–12 hours after collection. For a subset of animals, blood was processed for hematocrit and differential white blood cell (WBC) count (Work et al., 1998). Heterophil/lymphocyte (Gross and Siegel, 1983) and eosinophil/monocyte ratios were calculated from counts of 200 WBC per turtle.

Cell mediated immune status was evaluated as described (Work et al., 2000). Briefly, whole blood was diluted 1:2 in phosphate buffered saline (PBS), overlaid on an equal volume of Ficoll 1077 (Sigma, St. Louis, Missouri, USA) and centrifuged at 300 g for 15 min. Peripheral blood mononuclear cells (PBMC) were recovered from the Ficoll-saline interface, washed three times in PBS, and resuspended to a final concentration of 4×10^6 cells/ml in RPMI-1640 supplemented with 2-mercaptoethanol (0.005 M) and sodium pyruvate (3 μg/ml). Cell viability was assessed microscopically with Trypan blue dye exclusion (Sigma). Purity was determined by staining a film of the cell suspension with a Romanowsky-type stain (Leukostat, Fisher Scientific, Pittsburgh, Pennsylvania, USA), and classifying 200 cells under 1000× as mononuclear cells, granulocytes or red blood cells. Mean (±SD) viability of isolated PBMC on the day of culture was 88 ± 6% (range: 66–98%), and the percentage of PBMC was >95%. Cells were plated at 100 μl/well in 96-well flat-bottom microculture plates (Costar 3595, Corning Inc., Corning, New York, USA).

We used phytohaemagglutinin (PHA) and concanavalin A (ConA) (Sigma) as T-cell mitogens (Benjamini and Leskowitz, 1994; Cuchens and Clem, 1979a; Green and Cohen, 1979). We were primarily interested in evaluating T-like cell response because T-cells are thought to play a major role in tumor immunology (Greenberg, 1994). Mitogens were mixed with RPMI containing 0.5% (w/v) of Albumax-I (Gibco, Gaithersburg, Maryland, USA), a protein supplement, at concentrations of 1, 10 and 50 μg/ml for ConA and 0.1, 1, 10 and 100 μg/ml for PHA. Mitogens (100 μl/well) were dispensed in triplicate wells containing 100 μl of cells at 4×10^6 cells/ml. Triplicate wells containing 100 μl cells supplemented with 100 μl RPMI and Albumax-I with no mitogens served as unstimu-

lated controls for each assay. Final concentration of cells per well was 2×10^5; final concentration of mitogens per well ranged from 0.05 to 2.5 μg for ConA and 0.005 to 5 μg for PHA.

Plates were incubated for 90 hr in a humidified atmosphere at 32 C and 8% CO_2. Cells were pulsed with 3.7×10^4 Bq of tritiated thymidine (185 GBq/mmol) (Amersham Life Sciences Inc., Chicago, Illinois, USA) in 50 μl RPMI/protein supplement and harvested 18 hr later onto glass filters. Filters were placed in scintillation fluid, and radioactive decay (disintegrations/min [DPM]) was quantified using a liquid scintillation counter. Mean DPM of triplicate wells for each mitogen or antigen concentration was divided by mean DPM of triplicate control wells to calculate the stimulation index (SI). We used the peak SI of cultures containing different mitogen concentrations as the best indicator of the mononuclear cell proliferative response.

Humoral immune status was assessed using turtle plasma and a Beckman Paragon electrophoretic system with SPEP-II gels (Beckman Scientific, Fullerton, California, USA). Gels were electrophoresed, washed, and stained according to manufacturer instructions. Fractions (prealbumin, albumin, alpha 1, alpha 2, beta, and gamma globulin) were quantified by laser densitometry as percent of total protein. For best analysis, electrophoretograms were compared to known fractionation patterns of avian and mammalian species (Cray and Tatum 1998). Absolute values of each fraction (g/dl) were obtained using total protein measurements from a refractometer. The sum of albumins and globulins was used to calculate albumin/globulin (A/G) ratio.

Turtles were classified into 5 groups: Turtles from Kaneohe Bay with tumor scores 0 (KB0-no tumors), 1 (KB1), 2 (KB2), and 3 (KB3) (Work and Balazs, 1999), and non-tumored turtles from Kona/Kohala (Kona). Data for each group were summarized using means and standard deviation. To assess difference in body condition, slopes of curvilinear regression of weight to straight carapace length were compared. To test for significant differences between groups, we used one way analysis of variance (ANOVA) or non-parametric Kruskall Wallis ANOVA depending on whether data fit assumptions of normality or equal variance. Post-hoc pair-wise comparisons were done using Student Neumans or Dunn's test for significant differences with ANOVA or Kruskall Wallis test, respectively. To maintain an experiment-wide error rate of <0.05, we used a Bonferonni adjustment (alpha/n) where n was the number of analytes being compared (Rice, 1989). Spearman correlation was used to assess relationship between peak SI for ConA and PHA, and between gamma globulin levels and peak SI for ConA and PHA (Daniel, 1987). We used SAS (Cary, North Carolina, USA) to analyze slopes of weight and carapace length and Sigmastat (SPSS, Chicago, Illinois, USA) for all other statistical comparisons.

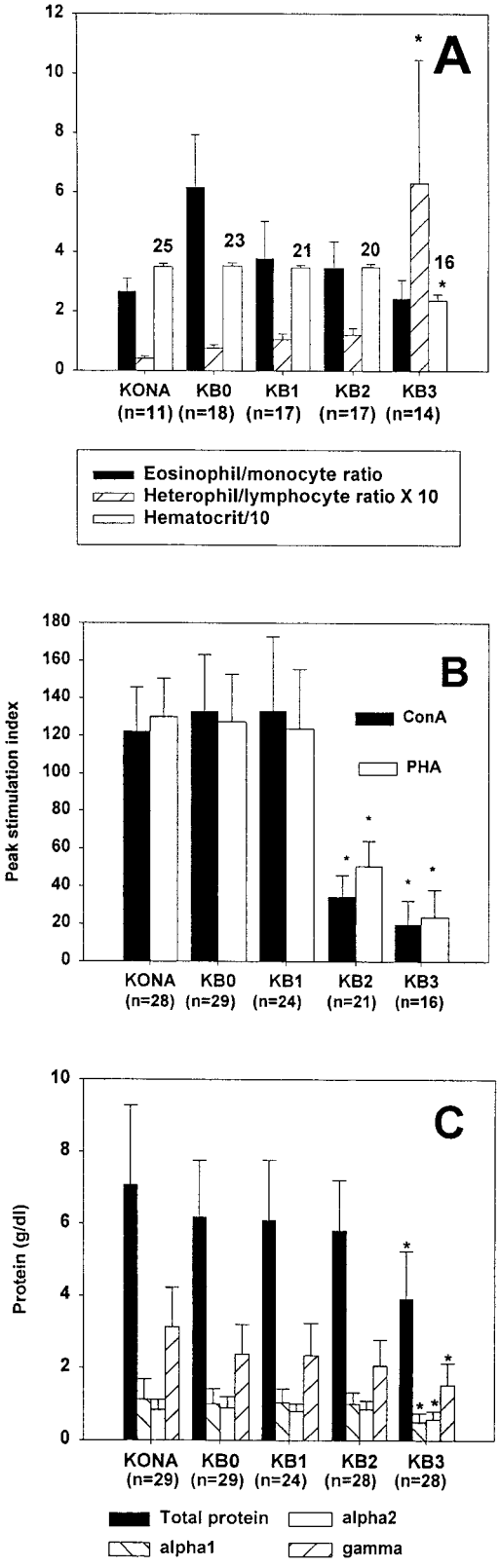
RESULTS

We sampled 120 turtles, 29 of which came from Kona/Kohala and the remainder from Kaneohe Bay. The Kaneohe Bay sample consisted of 29, 24, 21, and 17 turtles from tumor score categories 0, 1, 2, and 3, respectively. During the study, ocean temperatures in both sites ranged from 22–26 C. There was no significant difference between groups for slope of straight carapace length to weight.

Turtles in Kaneohe Bay showed a trend towards increasing heterophil/lymphocyte ratio and decreasing eosinophil/monocyte ratio with increasing tumor score (Fig. 1A). Tumor score 3 turtles from Kaneohe Bay had a significantly ($P < 0.084$) lower hematocrit and a higher heterophil/lymphocyte ratio than all other groups. There was no significant difference in hematological values obtained from non-tumored turtles from Kaneohe Bay and Kona/Kohala.

Proliferation results for ConA and PHA-stimulated turtle PBMC cultures were similar (Fig. 1B). Optimal mitogen concentrations for KB0 and Kona turtles were 0.1 to 1 μg/ml (0.05–0.5 μg/well) for ConA and PHA (data not shown). Significantly ($P < 0.001$) lower mitogen stimulation indices were seen for turtles with tumor scores 2 and 3 (Fig. 1B). No significant difference was seen between all other groups.

Turtles with tumor score 3 had significantly ($P < 0.004$) lower total protein, alpha 1, alpha 2, and gamma globulins (Fig. 1C); no significant differences were seen among all other groups. Albumin/globulin ratios and Beta globulins did not differ significantly between groups (data not shown). Relative percentages of protein fractions in plasma for KB0 turtles were



16, 14, 11, 39, and 20 for alpha 1, alpha 2, beta, gamma and albumin, respectively; these percentages did not differ significantly from those of Kona/Kohala turtles (16, 12, 10, 44, and 18). Prealbumin was seen only in eight turtles (0.02–0.18 g/dl).

Significant correlations were noted between peak SI between ConA and PHA ($r = 0.91$, $P < 0.001$) and between gamma globulin and peak SI with ConA or PHA ($r = 0.58$; $P < 0.001$).

DISCUSSION

Peripheral blood mononuclear cell proliferation assays indicated that tumor score 2 and 3 turtles had significantly impaired cell mediated immune status while non-tumored turtles from Kona/Kohala and Kaneohe Bay and tumor score 1 turtles were not significantly different. Varela (1997) reported decreased cellular proliferation in captive Florida green turtles with FP. However, he did not score the severity of disease nor did he clarify whether comparisons of tumored versus non-tumored animals was done with turtles from the same region. Mitogen stimulation of PBMC with PHA and ConA is used as an indicator of T cell-mediated mechanisms of immunity in a wide range of species, and there is considerable evidence for the existence of T and B like cells in reptiles and amphibians (Cuchens and Clem, 1979b; Manickasundari et al., 1984; Negm and Mansour, 1982; El Deeb et al., 1986; El Deeb and Saad, 1987). The correlation between proliferative responses to PHA and ConA for green turtle mono-

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FIGURE 1. Data (mean and SE) for turtles from Kona/Kohala (Kona) and turtles from Kaneohe Bay with tumor scores ranging from 0 (KB0) to 3 (KB3); asterisk indicates values that are significantly ($P < 0.004$) different. Sample size (n) is indicated below X-axis labels or above bars. Heterophil/lymphocyte ratio multiplied by 10, eosinophil/monocyte ratio, and hematocrit (%) divided by 10 (A). Peak stimulation indices for ConA and PHA (B). Total protein, alpha 1, alpha 2, and gamma globulin (g/dl) (C).

nuclear cells suggested we were working with a single subpopulation of cells analogous to PHA-inducible T-like lymphocytes of alligators (Cuchens and Clem, 1979a) or thymus dependent ConA- or PHA-inducible cells in frogs (Green and Cohen, 1979).

Possible reasons why cells of severely tumored turtles failed to proliferate include poor survivability in culture, inability to respond to mitogens, difference in mononuclear cell populations or subsets, deficient production of growth factors, or hypersensitivity to suppressor monocytes (Longo and Broder, 1988). Given that starting conditions for cell cultures were the same for all turtles (equal number of cells per well, similar cell viability and purity), it is unlikely that methodology differences were responsible for differences in proliferation seen here. Furthermore, optimal mitogen concentrations for PBMC of free-ranging non-tumored turtles were similar to those used in PBMC from captive non-tumored turtles (Work et al., 2000), again suggesting consistency in methodology.

It is also unlikely that seasonal variation in reptilian immune response (Nelson and Demas, 1996) accounted for the differences between tumored and non-tumored animals. First, Hawaii does not have the temperature extremes seen in more temperate areas of the United States thus making a distinction between winter and summer somewhat artificial. Second, tumored and non-tumored animals were assayed throughout the study period, and differences in proliferation assay results between tumored and non-tumored animals for all tumor score categories were consistent for both PHA and ConA (data not shown).

Differences in hematological parameters were evident only in the most severely tumored turtles (tumor score 3). Significantly increased heterophil/lymphocyte ratios were consistent with chronic stress in reptiles associated with elevated cortisol levels (Saad, 1988; Campbell, 1996) and were similar to observations of tumored green turtles from Hawaii (Aguirre et al., 1995; Work and Balazs, 1999) and Florida (Varela, 1997). Although it would have been preferable to compare absolute numbers of white cells rather than ratios of cell types, the trend in hematology versus tumor score seen in green turtles from Kaneohe Bay was similar to that of green turtles from Molokai, Hawaii (USA) where absolute cell counts were done (Work and Balazs, 1999).

Hypoproteinemia was evident only in the most severely tumored turtles (tumor score 3), and A/G ratios indicated that hypoproteinemia was caused by decreases in both albumin and globulin. Varela (1997) noted decreased A/G ratios in captive tumored turtles from Florida attributable to increased gamma globulins. In contrast, protein profiles of severely tumored Hawaiian turtles in this study showed significant decreases in alpha 1, alpha 2 and gamma globulins. Aguirre et al. (1995) also noted hypoproteinemia in free-ranging Hawaiian turtles with FP however, Swimmer (2000) did not.

Protein fractions in green turtles await further characterization, however, the molecular weight of proteins such as albumins and globulins in different animal groups (birds, mammals, reptiles) are remarkably similar (Kaneko, 1999). In mammals, decreases in alpha globulins are attributed to malnutrition, nephrotic syndrome, or liver disease (Kaneko, 1999). Based on emaciated appearance of some severely tumored turtles, we suspect that poor body condition was partly responsible for hypoproteinemia. Based on decreased gamma globulins, it is likely that B cell function and humoral immune status was also depressed in severely tumored turtles. In mammals, decreased gamma globulins are indicative of immunosuppression (Kaneko, 1999). The relative percentage of protein fractions in sea turtles, specifically albumin, was lower than that for terrestrial reptiles (Dessauer, 1970).

Hawaiian green turtles respond to se-

vere FP with hypoglobulinemia, lymphopenia, and decreased stimulation of PBMC with mitogens, all factors pointing to immunosuppression. On the other hand, lack of differences in hematologic or immunological parameters between tumor-free turtles from Kaneohe Bay, a FP-endemic site, and Kona/Kohala, a FP-free site, supports the hypothesis that non-tumored turtles from both tumor free (Kona/Kohala) and tumor-endemic (Kanehoe Bay) locations are equally immunocompetent. Lack of significant differences between immunological profiles of tumor score 1 turtles and non-tumored turtles on Oahu suggests that immunosuppression may be a consequence of fibropapilloma development and growth rather than a predisposing factor for tumor development. Further reinforcing this concept is the absence of evident cell mediated or humoral immunosuppression in a captive green turtle that was challenged with a standard dose of antigen and that subsequently developed FP (Work et al., 2000).

Several factors could explain depressed immune status in severely tumored turtles. First, viral-induced tumor diseases in other animals such as Mareks or SV40 of primates can induce immunosuppression (Thomson et al., 1988). Second, Aguirre et al. (1998) estimated that 96% of turtles with FP had concurrent vascular trematode infections, and vascular trematodes are known to be immunosuppressive (Sher and Ottesen, 1988). Third, previous pathologic examination of green turtles (Aguirre et al., 1998; Work and Balazs, 1998) revealed moderate to severe emaciation, cachexia, soft and sunken plastron, and a muddy decalcified carapace with algal growth indicating a reduced activity of tumored turtles in the wild. While this was also observed in this study, we were unable to quantify significant differences in body condition using curvilinear regression of weight on straight carapace length, perhaps because of insufficient sample size. The use of the ratio of weight to straight carapace length was not an option since it would have been confounded by the larger size of tumored turtles (data not shown). Furthermore, Jacobson et al. (1993) noted that this ratio was not a satisfactory method to assess body condition in desert tortoises. This points to a clear need to develop quantitative criteria to assess body condition in sea turtles.

Sorting out whether FP, infectious agents, or poor body condition are responsible for the immune changes seen here in tumored turtles would require us to evaluate immune status in turtles in poor body condition but free of tumors and infectious agents (herpes virus or vascular flukes). The lack of sufficient study subjects meeting these criteria and lack of a suitable method to quantify body condition in sea turtles makes this task impractical at this time. Furthermore, this study did not rule out the possibility of a selective impairment of components of the immune system such as cytokine production. While we showed a difference in immune status between free-ranging tumored and non-tumored turtles, it remains to be determined whether these differences are due to presence of infectious agents, factors in the environment, or factors within the turtles themselves. Future investigations might focus on the role of herpesvirus, vascular flukes, or biotoxins (Landsberg et al., 1999) in immune function, or on evaluating cytokine levels to obtain additional assessments of the immune status of green turtles.

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